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Introduction

The digitalis-like cardiac glycosides exert a number of different actions on the mammalian heart *in situ*. Among the more important pharmacological effects of these compounds on the myocardium are the changes which they produce in the conduction system of the heart and in the factors associated with contraction of cardiac muscle. In therapeutic dosage, the glycosides cause a slowing of impulse conduction in the heart and bring about increased force of cardiac systolic contraction accompanied by a decrease in diastolic size. These manifestations of pharmacological activity are present in both the human being and in the experimental animal, in both the normal state and in either experimentally induced cardiac dilatation or in the failing human heart in clinical cardiac decompensation. It is generally accepted that the greatest portion of the effects just outlined are due to direct action of the drugs on the conduction bundle and myofibrils of the myocardium. Any property of a drug attributed to its "direct action" on a given tissue could conceivably be associated with an effect of the drug on the metabolism of the cells composing the tissue. That such a possibility might be responsible for some of the observed responses of the heart to the cardiac glycosides was considered plausible from several viewpoints. The viewpoints considered involve speculation relative to the possible effects of these compounds on three enzyme systems present in heart muscle.

The work of Szent-Györgyi and his many co-workers and collaborators indicates that optimum levels of adenosine triphosphate (ATP) are necessary for optimal contraction of striated muscle.[1,2] The work of Rozsa[3] indicates that, in the case of the active contractile element of heart muscle (heart actomyosin), the same necessity for an ATP optimum exists for optimal contraction. Rozsa also showed that the actin and myosin of striated, smooth, and heart muscle of the rabbit are interchangeable, actin from one giving contractile actomyosin with the myosin of the other and vice versa. Further indication that cardiac muscle requires critical levels of ATP for optimal contraction can be drawn from the work of Chang[4a] and of Burns and Cruckshank.[5] These investiga-

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tors demonstrated decreased efficiency of contraction of the heart under conditions (*i.e.*, graded levels of iodoacetate poisoning) that are now known to diminish ATP synthesis. Chang has also shown that during anoxia the mechanical efficiency of the rabbit heart is qualitatively correlated with the ATP level.[4b] Work in the laboratories of this institution directed by the late Professor A. G. Mulder (with the assistance of the authors and others) has demonstrated a significant decrease in the ATP content of *in situ* dog hearts in experimentally induced pathological states as compared with normal *in situ* control dog hearts.[6] It is conceivable that the decreased mechanical efficiency observed in the hearts examined in those experiments might be correlated with the decreased ATP levels found. It is also possible that at least a part of the mechanism of the action of the cardiac glycosides might reside in their effect on the enzyme systems that regulate the level of ATP in heart muscle.

One of these enzymes, adenosine-triphosphatase (ATPase), is responsible for hydrolysis of ATP in muscle. Within the scope of our present knowledge (with one exception to be discussed later), it would seem that the balance between the ability of the carbohydrate metabolism to produce ATP and the activity of ATPase is the factor governing the level of ATP in the myocardium. Barron and Singer have shown that ATPase is a sulfhydryl critical enzyme (*i.e.*, requires free sulfhydryl groups for its activity).[7] This fact, considered along with certain aspects of the chemistry of the cardiac glycosides, makes a selective inhibition of ATPase very possible. Digitalis-like glycosides contain an unsaturated lactone ring at C-17 in the steroid moiety of the molecule, the integrity of which is essential to cardiac activity.[8] The lactone ring contains a conjugated carbon-carbon double bond carbonyl system. It is well known that many compounds with this critical structure have sulfhydryl reactivity, that they effect addition of sulfhydryl compounds, and that factors relative to chemical structure and reaction conditions determine whether or not these reactions occur.[9] It has also been demonstrated that the free lactone compounds with this structure are capable of forming peroxides in the presence of dissolved molecular oxygen.[10,11] This peroxide formation has been shown to increase the cardiotonic activity of the lactones.[11,12] As peroxides are quite capable of sulfhydryl reactivity,[9] the possibility that a glycoside lactone ring peroxide formation may yield a structure capable of inhibiting ATPase activity by oxidizing enzyme sulfhydryl groups cannot be overlooked.

The presence of critical sulfhydryl groups in ATPase and the presence of a sulfhydryl reactive unsaturated lactone ring in the cardiac glycosides presents the possibility of a glycoside produced oxidation of ATPase sulfhydryl yielding inhibition of the enzyme and preservation of

ATP. Such an inhibition would foster increased efficiency of cardiac contraction by this sparing of ATP, which, in several pathological heart states[4b,5,6] may be at a low level due either to lowered rate of synthesis or abnormally increased rate of destruction or both. Preliminary experiments carried out by one of the authors seem to indicate that administration of calculated therapeutic doses of digitoxin to dogs results in a rise in the adenosinepolyphosphate (APP) content of the heart as compared with APP levels in undigitalized control dog hearts. While the series studied is not as yet sufficient in size to establish significance, such results, considered along with the known characteristics of ATPase and the chemical structure-activity relationships of the cardiac glycosides, tend to support the possibility that the glycosides may exert part of their pharmacological action on the heart by selectively inhibiting ATPase. It is interesting to note that Kimura and DuBois have already demonstrated an inhibition of rat heart ATPase by ouabain, albeit the ouabain concentrations used by them are considerably greater than those achieved in the heart *in vivo.*[13]

Still another enzyme system involved with alteration of ATP structure is worthy of consideration. Snellman and Gelotte have reported the presence of a contamination in heart actin which deaminates ATP.[14] While this deaminase was sufficiently active to prevent the polymerization of the actin to a very great extent, the presence of a cardiac glycoside could allay the effect of the enzyme. Banga and Josepovits have also reported the presence of this enzyme,[15] but the authors have been unable to locate any detailed characterization of it in the literature. It is immediately apparent that this enzyme could affect the balance between ATP production and ATP destruction and, hence, the level of ATP in heart muscle. The necessity of ATP for preservation of the functional integrity of actin, myosin, and actomyosin has been well established.[1,2] Since the function of these integral components of the heart muscle contractile element is critical to optimum contraction, the observed inhibition of this enzyme by a cardiac glycoside must necessarily be considered in any hypothesis on the mechanism of cardiac glycoside action. As deaminases have usually been shown not to be sulfhydryl critical, a glycoside effect on such possible enzyme prosthetic groups was considered unlikely though not excluded. It is well established that many deaminase systems require oxygen as one of their reactants. Certain polarographic observations made by one of the authors have indicated cardiac glycoside complexing of dissolved oxygen.[16] This finding has posed two questions to the authors, *i.e.*, as to whether or not the ATP deaminase requires molecular oxygen for activity, and, if it does, whether or not the cardiac glycosides, by complexing this

reactant, prevent deaminase access to it to account for inhibition of enzyme activity.

In many respects, the actions of cardiac glycosides on the conduction system of the heart are very similar to those elicited by acetylcholine. For example, the glycosides cause generalized slowing of conduction and atrioventricular nodal delay with very little, if any, prominent action on sinoauricular nodal tissue. The action of acetylcholine is exactly analogous. The release of intracellular potassium caused by digitalis [17] and the increased susceptibility of stropanthanized animals to acetylcholine has turned attention to the possibility of cardiac glycoside antiacetylcholinesterase activity. In dogs, pretreated with strophanthin, the hypotensive and cardiac actions of acetylcholine are greatly enhanced, [18] and it is well known that acetylcholine causes a release of muscle cellular potassium. [19,33] Acetylcholinesterase (ACHase), like ATPase, contains sulfhydryl groups believed to be critical for its activity. It is therefore possible that the cardiac glycosides could function with reference to the heart ACHase in a manner analogous to that which has been postulated for ATPase. Such inhibition of ACHase would decrease the lability of endogenous heart acetylcholine, would increase its effective concentration, and may thereby afford part of the explanation for the slowed conduction time seen in hearts under the influence of the cardiac glycosides.

The observations outlined in the preceeding paragraphs have caused the authors to proceed to an evaluation of the effects of digitoxin on the heart ATPase, ACHase, and ATP deaminase of the rabbit. This animal was selected because concentration ranges for therapeutic and toxic heart tissue levels of cardiac glycosides have been established for it. [20] In the present investigation, *in vitro* studies have been undertaken because it was felt that such conditions afforded the best possibility of testing the fundamental mechanisms that have been postulated. Polarographic studies designed to analyze the effects produced by digitoxin have been performed and correlated with enzyme studies where indicated.

Methods Used for Study of Heart ATPase and ACHase

ATPase activity of heart muscle homogenates was determined by a method essentially like that used by Bell, Carr, and Krantz to determine arterial wall ATPase activity.[21] The method of Griswold, Humoller, and McIntyre was used to determine the phosphate liberated. [22] Phosphate spectrophotometer readings were taken at 660 mμ rather than at the 450 mμ wave length which they used. ACHase activity was measured in a manner similar to that used by Diggle and Gage for the brain.[23] Hestrin's method was used to determine the residual acetylcholine.[24] In the case of both of these enzymes, variations in organ enzyme concentration

exist, and modifications in the homogenate levels for other tissues used by the original workers[21,23] had to be made in order to effect measurable enzyme action within limits imposed by the enzyme levels available in heart muscle. In the ATPase determinations, sodium chloride-glycine buffer (pH 7.4) was used instead of a veronal buffer[21] so as to exclude any possible effect due to the barbiturate.

Sacrifice of the rabbits used was accomplished by administering a blow to the back of the head. The heart was then excised in a matter of seconds and washed in cold distilled water to remove the blood. Following removal of the atria and superficial fat, the ventricles were blotted dry with filter paper and weighed on a torsion balance to the nearest tenth of a gram. The tissue was then ground in cold sand with a previously chilled mortar and pestle and finally homogenized in the mortar with sufficient distilled water to make a 30 per cent (w/v) homogenate of the tissue. This homogenate was usually centrifuged for five minutes at 580 $\times$ g. to yield a distinctly turbid supernatant fluid (in some experiments on ATPase, the centrifugation was carried out at 900 $\times$ g.). Two ml. aliquot portions of this supernatant were used to prepare control and drug-treated sources of enzymes. These aliquots were placed in test tubes in a constant temperature water bath set at 37° C. $\pm$ 0.1° C. In the majority of the experiments, digitoxin, 5 μg. contained in 0.2 ml. of 40 per cent ethyl alcohol, was added to one 2-ml. aliquot of the homogenate, while an equal volume of 40 per cent ethyl alcohol was added to another 2-ml. aliquot of homogenate which served as a control.* The tubes were allowed to incubate for 30 minutes in the water bath. At completion of this incubation period, aliquots of the 30 per cent homogenates were taken and employed directly in determinations of ACHase activity, and, following dilution to 2 per cent homogenate concentration, were employed in the reaction mixture used for estimation of ATPase activity. Changes in digitoxin concentration, incubation time, additions of other reagents such as sulfhydryl compounds, *etc.* have been made according to the needs of experimental design. Such changes, as well as the specific conditions used to study these two enzyme reactions in the various experiments undertaken in this investigation have been presented in table footnotes and figure legends.

Methods Used for Study of Heart ATP Deaminase

Polarographic studies undertaken by one of the authors have revealed that ATP and other adenine derivatives yield a polarographic wave in

*In the early experiments, an additional control, consisting of a 2-ml. aliquot of homogenate to which 0.2 ml. of distilled water had been added, was run. As enzyme reactions obtained using this homogenate did not differ appreciably from those obtained using homogenate that contained the digitoxin solvent (0.2 ml. of 40 per cent ethyl alcohol) it was deemed unnecessary to continue running such an additional control.

perchloric acid supporting electrolyte that is not given by inosine or any of its common derivatives, including inosine triphosphate (the most likely product of ATP deamination). None of the other 15 pyrimidine and purine derivatives tested thus far have shown this reduction. As the polarographic waves yielded by ATP under these conditions have wave heights that are proportional to ATP concentration, and since the waves obtained seem to be characteristic for amino substitution on the 6- position of a purine nucleus with an unsubstituted 2- position (*i.e.*, no waves are given by either hypoxanthine or cytosine), it was realized that polarography had afforded a good analytical tool for estimation of ATP deaminase activity. This is made even more apparent when one considers the fact that inosine derivatives can interfere with spectrophotometric estimation of adenine to some extent.[25] As a result of these findings, polarography has been used to determine the adenine moiety of the ATP used in ATP deaminase reaction mixtures.

The work of Horvath and his co-workers,[26] designed to study the effects of a cardiac glycoside on dog heart actin polymerization, gave indication that potassium and magnesium might be ions required for optimal ATP deaminase action. Because of this, ATP deaminase enzyme reaction mixtures were designed containing these ions. Reaction mixtures containing sodium and calcium were also studied. Indication of specific reaction conditions in table footnotes, the procedure followed in the case of the ATPase and ACHase studies, has also been used in presenting data from this part of the study.

The residual ATP left after deamination was determined by polarographic analysis in the following manner: after cessation of the enzyme reaction by addition of either 0.2 ml. of 0.1 N sodium acetate saturated with ammonium sulfate or 0.2 ml. of 10 per cent silicotungstic acid to the enzyme reaction mixture, the solutions were filtered. One ml. aliquots of this filtrate were removed by pipette and added to 5 ml. of 0.2 N perchloric acid. The volume was adjusted to 10 ml. with distilled water, the solution deaerated with nitrogen for 5 minutes, and the polarogram taken at voltage settings fixed to include wave of ATP. This wave has a half-wave potential of -1.04 V versus the Hg pool in the cases where the saturated ammonium sulfate in 0.1 N sodium acetate is used as protein precipitant, and a half-wave potential of -1.05 V versus the Hg pool in the cases where 10 per cent silicotungstic acid is used for the same purpose. Separate ATP polarographic calibration curves are required for each of the protein precipitants due to the alteration in final polarographic supporting electrolyte which they cause.

The preparation of the homogenates (both control and drug treated) was accomplished in the same manner as that used in the ATPase studies,

except that, in most instances, the homogenate concentration used in the enzyme reaction mixtures was 10 per cent (w/v). The incubation period of the glycoside with the homogenate was identical to that (30 min.) used for the ACHase and ATPase procedures. In the majority of the experiments, studies on the three enzymes were carried out simultaneously, using the same control and drug-treated homogenate sources. In the case of ATP deaminase, determination of the inorganic phosphate content of the reaction mixture filtrates was also carried out using the method of Griswold, *et al.*[22] This was done in order to determine whether or not the deamination was effected on ATP per se and not on one or the other of its hydrolysis products such as adenosinediphosphate (ADP).

Polarographic Methods

The present investigation is not primarily concerned with either theoretical or applied polarography. Consequently most of the detailed information on such studies relating to this work will be published elsewhere. Only the general polarographic procedures used are outlined here. Specific conditions believed to be germane to an understanding of the results and the discussion have been entered in either table footnotes or figure legends.

All polarographic determinations have been carried out at 25° C. ± 0.1° C. in order to take advantage of known polarographic constants. The dropping mercury electrode served as cathode with reference to the mercury pool as anode in all cases. The Sargent model XXI recording polarograph was used to record the polarograms.

The possibility that cardiac glycosides might form peroxides similar to those formed by their analogous free lactone ring substituent[10] was investigated by use of polarography. Digitoxin was added to various air-saturated buffer solutions (pH 7.4) in a closed system, and the oxygen level of the solutions was observed polarographically[27] at frequent time intervals. This was continued until several successive constant polarograph readings for oxygen had been obtained to indicate cessation of oxygen disappearance from the solution. Control solutions corrected for the volume change due to addition of the glycoside solution on the one hand and for the presence of the glycoside solvent (ethyl alcohol) on the other hand were run simultaneously. No difference between the rate of oxygen disappearance from the two control solutions was observed in the case of any of the buffers used. The effect of different metallic ions on the rate of oxygen uptake by digitoxin solutions was also studied. Salts of these metals were added to both digitoxin and control buffer solutions in such experiments. After several successive constant oxygen levels had been observed in the buffer solutions containing digitoxin, these solutions were deaerated with nitrogen for 15 minutes, and polarograms

were taken on them in the voltage range usually found for the reduction of peroxides.[10,27]

Polarographic studies made on the status of sulfhydryl content in some of the control and drug-treated homogenates have been made possible by preliminary work undertaken by one of the authors on the polarography of p-chlormercuribenzoate.[28] It has been ascertained that the first of the two polarographic waves given by this mercaptide forming reagent in 0.15 M phosphate buffer (pH 7.4) is quantitatively reduced in height by the presence of sulfhydryl substances such as cysteine. This effect is not produced by substances that contain only disulfide linked sulfur (*i.e.*, insulin, cystine) or ethereal sulfur (*i.e.*, methionine), nor has any such effect been noted in the case of any of the several sulfur-free amino acids tested thus far. It was felt that this observation afforded opportunity to check the effect of the digitoxin on the status of homogenate sulfhydryl groups. In experiments in which this has been studied, aliquots of the homogenate solutions have been taken and diluted appropriately so as to come within the range of the method. Equivalent aliquots of the diluted homogenates (control and drug treated) were then

Table 1

Effect of Digitoxin on Rabbit Heart ACHase Activity*

Experiment No.	μM acetylcholine hydrolyzed/30 min.		Per cent inhibition due to digitoxin
	Control	Digitoxin**	
1.	15.2	6.9	55
2.	18.4	11.5	38
3.	13.8	9.2	41
4.	18.4	13.8	25
5.	9.2	4.6	50
6.	18.4	9.2	50
7.	16.1	9.2	43
8.	13.8	10.6	23
9.	20.7	16.1	22
10.	11.5	6.9	40
11.	11.5	7.7	33
12.	11.5	7.7	33
13.	16.1	9.2	43
14.	13.8	6.9	50
15.	16.1	9.2	43

*Enzyme reaction time was 30 minutes at 37° C. Enzyme reaction mixtures were made up according to the following formula: 1 ml. 0.45 M NaCl; 1 ml. 0.075 M $NaHCO_3$; 0.5 ml. 0.24 $MgCl_2$; 1 ml. 0.012 M acetylcholine bromide and 1 ml. of 30 per cent homogenate. Controls for spontaneous hydrolysis were run in which 1 ml. of distilled water was added in place of the homogenate. Each result is the average of two duplicate determinations.

**Digitoxin was added to the 30 per cent homogenate to effect a concentration of 2.5 μg./ml. and incubated 30 minutes before addition to enzyme reaction mixtures. Control homogenates were incubated under the same conditions.

added to p-chlormercuribenzoate solution in buffer, deaerated with nitrogen for 10 minutes, and polarograms were taken on the solution at voltage settings adjusted to include the first p-chlormercuribenzoate wave. This wave has a half-wave potential ($E\frac{1}{2}$) at -0.28 V versus the Hg pool. Any reduction in the first wave-height of the mercury compound (as compared with a control of equivalent p-chlormercuribenzoate concentration) was ascertained, and the sulfhydryl content (as cysteinyl units) was estimated by comparison with a calibration curve obtained using known concentrations of cysteine to bring about wave-height reduction.

Results

The data from the experiments presented in TABLES 1 and 2 indicate that digitoxin inhibition of rabbit heart ACHase and ATPase was obtained consistently in the experiments performed. The per cent inhibition of ACHase was qualitatively greater than that observed in the case of ATPase. The analysis for the degree of reversibility involved in the inhibitions was accomplished according to the method of Ackermann and Potter.[29] The results obtained are illustrated in FIGURES 1 and 2. They

TABLE 2

EFFECT OF DIGITOXIN ON RABBIT HEART ATPASE ACTIVITY*

Experiment No.	ATPase units**		Per cent inhibition due to digitoxin
	Control	Digitoxin***	
1.	0.77	0.55	29
2.	1.75	1.67	4
3.	1.85	1.65	10
4.	1.10	0.85	23
5.	1.73	1.43	17
6.	0.90	0.80	11
7.	1.43	1.33	7
8.	1.53	1.05	31
9.	1.43	1.20	15
10.	1.35	1.10	18
11.	1.35	1.25	7
12.	1.40	1.18	16
13.	0.98	0.90	7
14.	0.98	0.83	15

*Enzyme reaction time was 15 minutes at 37° C. Enzyme reaction mixtures were made up according to the following formula: 1.2 ml. 0.1 M NaCl-glycine buffer, pH 7.4; 0.5 ml. 0.032 M $CaCl_2$; 1 ml. 0.010 M ATP, Na salt; 1.1 ml. distilled water, and 1 ml. of 2 per cent homogenate. Controls for spontaneous hydrolysis were run in which 1 ml. of distilled water was added instead of the homogenate. Each result is the average of two duplicate determinations.

**ATPase unit = μg. of phosphate liberated by 1 mgm. of tissue in 15 minutes.

***Digitoxin was added to an original 30 per cent homogenate to effect a concentration of 2.5 μg./ml. and incubated 30 minutes before dilution to the 2 per cent level and immediate addition to the enzyme reaction mixtures. Control homogenates were incubated and diluted under the same conditions. The undiluted (30 per cent) homogenate was centrifuged at 900 × g. in experiments 1 to 5 and at 580 × g. in experiments 6 to 14.

indicate that the complex formation between digitoxin and ACHase is either irreversible or pseudoirreversible, while the complexing between the glycoside and ATPase is reversible. Analysis of the digitoxin ATPase inhibition, according to the method of Hunter and Downs,[43] indicates that the inhibition is competitive and is therefore subject to regulation by the substrate concentration (see FIGURE 3).

Preliminary experiments have been carried out by the authors to ascertain some of the characteristics of the heart ATP deaminase described in a general way by others.[14,15,26] These experiments indicated

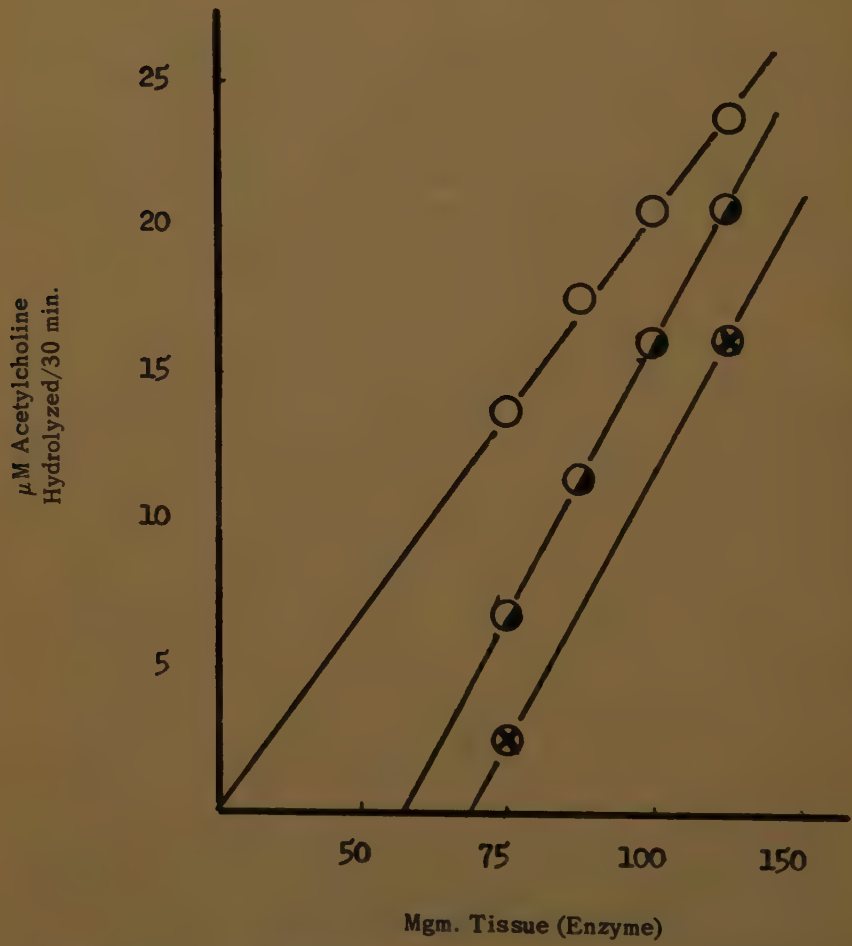

FIGURE 1. Analysis of digitoxin inhibition of ACHase according to the method of Ackerman and Potter.[29] Open circles are controls; half-closed circles indicate digitoxin present at 0.6 micromolar final concentration; crossed circles indicate digitoxin present at 1.2 micromolar final concentration.

optimal enzymatic activity when both magnesium and potassium were present in the enzyme reaction mixture. Enzymatic activity was demonstrated in the presence of calcium and sodium ions either when together or in combination with either magnesium or potassium separately. However, lower activity than that seen when both potassium and magnesium were present together was demonstrated in the case of these other ion combinations. Consequently, most of the experiments performed on ATP

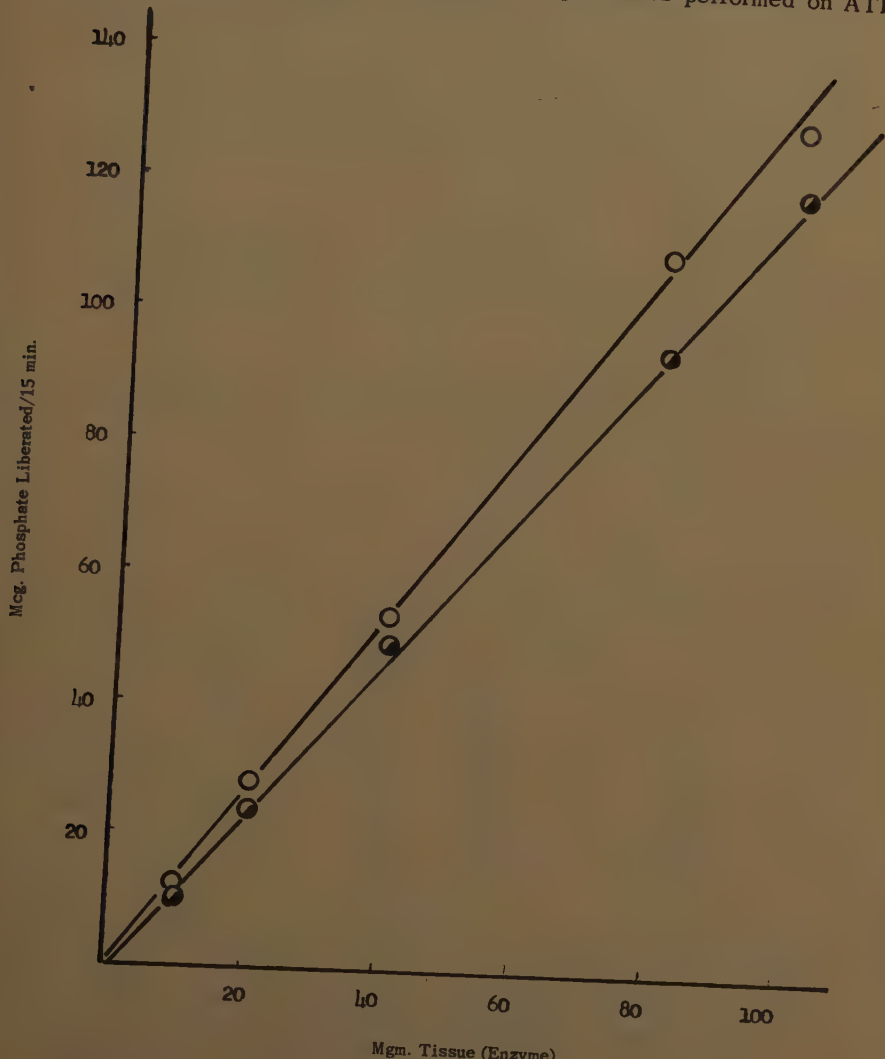

FIGURE 2. Analysis of digitoxin inhibition of ATPase according to the method of Ackerman and Potter.[29] Open circles are controls; half-closed circles indicate digitoxin present at 2.4 micromolar final concentration.

deaminase have been undertaken in KCl-glycine buffer (pH 7.4) in the presence of magnesium ion. The results listed in TABLE 3, experiments 1 to 8, indicate that consistent inhibition of this enzyme was effected by digitoxin. Since the phosphate liberated in these experiments (due in all probability to magnesium catalyzed-ATPase activity) is insufficient in amount to account adequately for the deamination of the hydrolysis products of ATP, the data indicate that a true ATP deaminase exists in rabbit heart with ATP as its specific substrate. The oxygen dependance of the deamination was established in experiments 9 through 12, TABLE 3, where it was demonstrated that while no deamination took place in nitrogen-saturated enzyme reaction mixtures (experiments 11 and 12), enzyme activity was quite evident in the mixtures having an air gas phase (experiments 9 and 10).

Polarographic investigation of the effect of digitoxin on oxygen has revealed several interesting phenomena. These findings, which have been demonstrated in three different buffer systems, all at pH 7.4, will be described in terms of the results obtained in one buffer (0.1 M KCl-glycine) for sake of brevity.

Early in the course of the investigation, it was ascertained that the half-wave potential ($E\frac{1}{2}$) of oxygen in buffer solutions was shifted to a

TABLE 3

EFFECT OF DIGITOXIN ON RABBIT HEART ATP DEAMINASE ACTIVITY*

Experiment No.	μM ATP deaminated/15 min.		μM PO_4 released/15 min.		Per cent inhibition of deaminase
	Control	**Digitoxin	Control	**Digitoxin	
1.	2.20	0.70	1.03	0.96	67
2.	1.16	0.44	1.21	1.02	62
3.	2.20	1.20	1.03	0.96	45
4.	2.30	0.38	1.53	1.48	83
5.	8.36	4.18	1.21	1.10	50
6.	2.30	0.38	1.53	1.48	83
7.	8.36	4.18	1.21	1.18	50
8.	8.36	4.90	1.21	1.31	41
9.	3.32	--	1.48	--	--
10.	2.39	--	1.36	--	--
11.***	0.00	--	1.50	--	--
12.***	0.00	--	1.32	--	--

*Enzyme reaction time was 15 minutes at 37° C. Enzyme reaction mixtures were made up according to the following formula: 1.2 ml. 0.1 M KCl-glycine buffer, pH 7.4; 0.5 ml. 0.032 M $MgCl_2$; 1.0 ml. 0.0144 M ATP, Na salt; 1.1 ml. of distilled water and 1 ml. of 10 per cent homogenate. Controls for spontaneous changes in ATP were run in which 1 ml. of distilled water was added in place of the homogenate.

**Digitoxin was added to an original 30 per cent homogenate to effect a concentration of 2.5 μg./ml. and incubated 30 minutes before dilution to the 10 per cent level and immediate addition to the enzyme reaction mixtures. Control homogenates were incubated and diluted under the same conditions.

***The gas phase was nitrogen in experiments 11 and 12.

more negative potential in the presence of digitoxin. As such a shift is often a manifestation of complexing action, the effect of change in digitoxin concentration on the $E\frac{1}{2}$ of oxygen was studied. An example of the results of such studies is presented in FIGURE 4, where the half-wave potential ($E\frac{1}{2}$) is plotted against the log digitoxin concentration (log C_x) to yield a straight line. Such a plot indicates complexing and allows for a determination of the complex number, *p*, by use of the

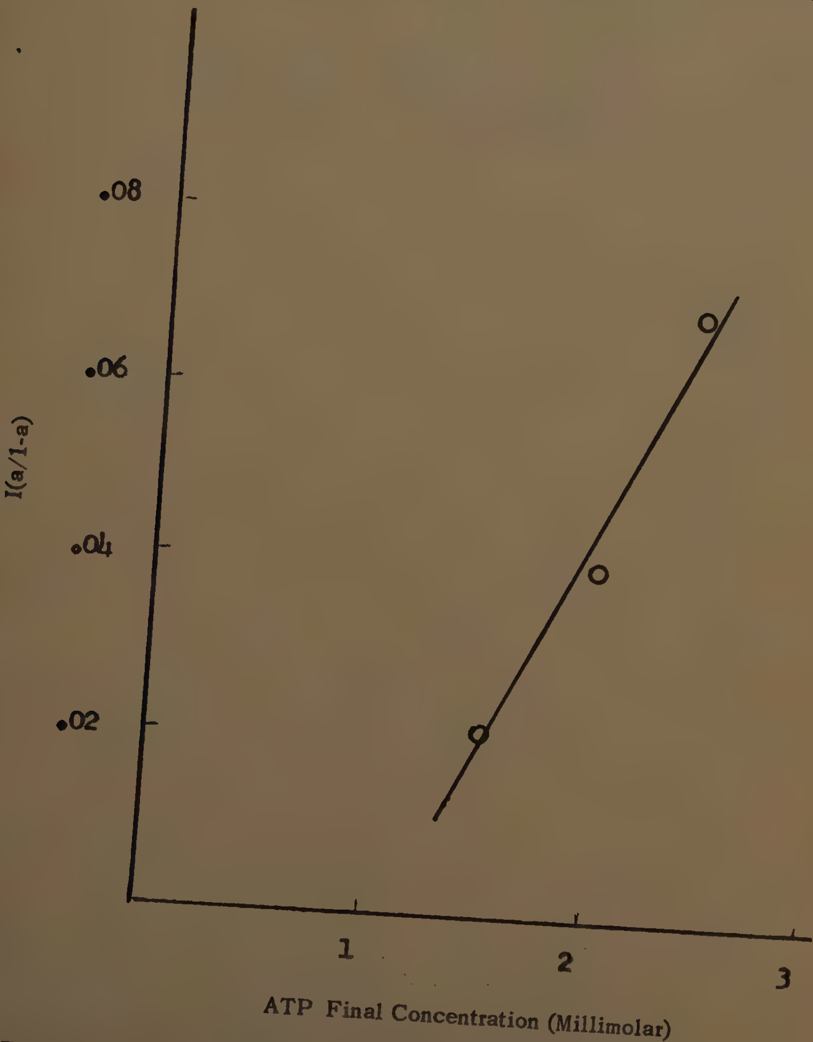

FIGURE 3. Analysis of digitoxin inhibition of ATPase according to the method of Hunter and Downs.[43] The final digitoxin concentration is 5 micromolar in this experiment.

following well-known equations,[27] in which n is the number of electrons involved in the reduction:

(1)
$$\frac{\Delta E\frac{1}{2}}{\Delta \log C_x} = \frac{-p(0.0591)}{n}$$

(2)
$$-p = \frac{\Delta E\frac{1}{2}}{\Delta \log C_x} (n/0.0591$$

Calculation of p from the slope of the surve in FIGURE 4 gives a complex number of 4, indicating in all probability that four molecules of digitoxin complex one molecule of oxygen. As the isoaglycone of digitoxin failed to give a shift in oxygen $E\frac{1}{2}$, it seems safe to assume that

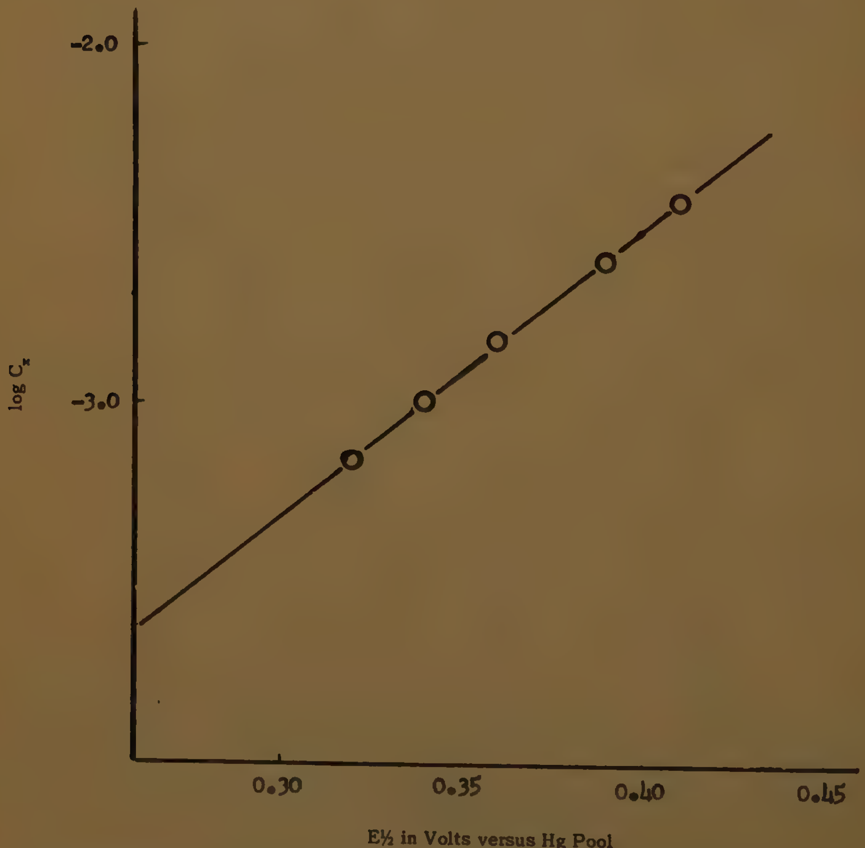

FIGURE 4. Polarographic demonstration of digitoxin complexing effect on oxygen.

the complexing occurs at the site of the digitoxin lactone ring, the structural integrity of which is no doubt necessary for the manifestation of the phenomenon.

The results of polarographic experiments designed to determine whether or not the digitoxin takes up dissolved oxygen from solution seem to indicate that the glycoside does possess this property. Qualitatively, the oxygen uptake occurs at a greater rate in KCl-glycine buffer than in either NaCl-glycine or veronal buffers. Magnesium ion accelerated the rate while calcium, cobalt, and manganese ions failed to affect the rate when present at concentrations equivalent to the magnesium levels (0.024 molar) used. The total digitoxin uptake of oxygen was not stoichiometrically related to the amount of glycoside present in the

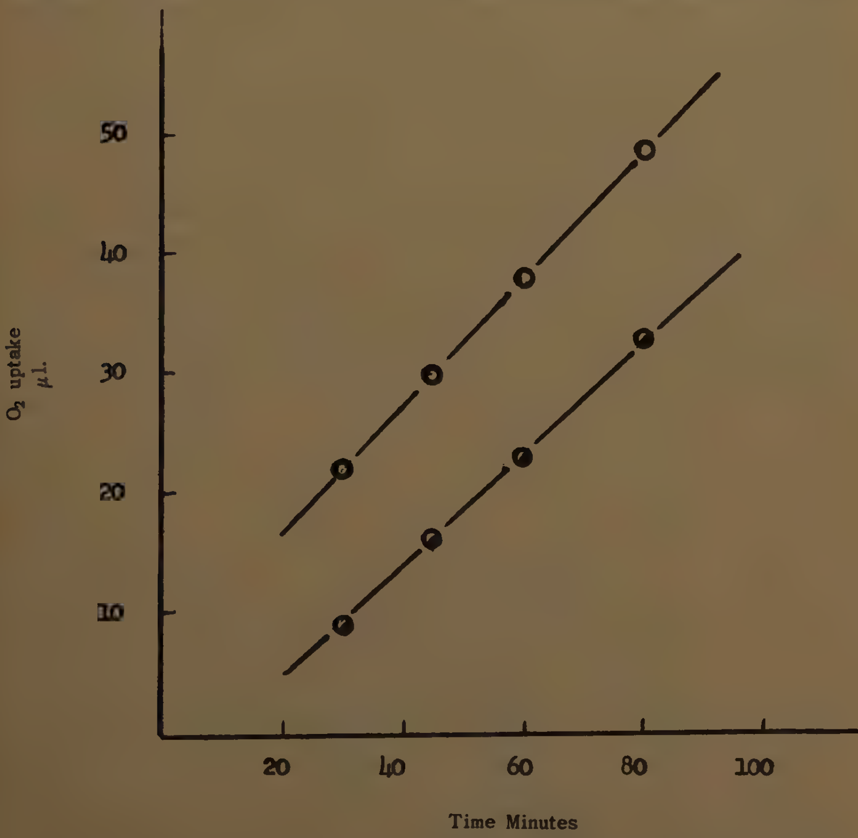

FIGURE 5. Oxygen uptake by digitoxin as measured polarographically. Half-closed circles indicate 3.5 micromoles digitoxin in KCl-glycine buffer; open circles indicate 3.5 micromoles of digitoxin in KCl-glycine buffer containing diluted whole rabbit blood at a final dilution of 1:100,000. The pH was 7.4 in both cases. All results are corrected for the analogous control oxygen uptake.

solution but averaged about 40 per cent of the equivalent uptake theoretically possible in terms of a mole-to-mole reaction. A similar phenomenon has been observed by others in the case of oxygen uptake by the free angelica lactones.[10] FIGURE 5 illustrates the rate of digitoxin uptake of oxygen in KCl-glycine buffer in the presence of magnesium ion. It also shows that inclusion of diluted whole rabbit blood in the system considerably accelerates oxygen uptake by the glycoside.

Solutions in which oxygen uptake by the glycoside had reached an apparent limit were deaerated, and polarograms were run on them in the potential range usually found for reduction of peroxides. A wave was located with an $E\frac{1}{2}$ of -1.42V versus the Hg pool in KCl-glycine buffer. Addition of hydrogen peroxide to the solution followed by a re-run of the polarogram revealed that the wave was distinct from that yielded by hydrogen peroxide, the latter wave having an $E\frac{1}{2}$ at -0.9 V versus the Hg pool. The possibility that digitoxin might be complexing hydrogen peroxide was ruled out by control experiments in which polarograms taken on deaerated solutions containing these two substances in buffer revealed no alteration in the hydrogen peroxide wave position or height. These findings, along with the negative result obtained when production of the -1.42 V-$E\frac{1}{2}$ wave was attempted in digitoxin-buffer solution saturated with nitrogen, have caused us to reach the conclusion that the wave observed represents a product resulting from digitoxin uptake of oxygen. Subjection of the wave to theoretical polarographic analysis[27] has revealed that it is characteristic of the polarographic reduction of a peroxide.

TABLE 4

COMPARATIVE EFFECT OF DIGITOXIN AND "PEROXIDE" DIGITOXIN ON RABBIT HEART ATPASE ACTIVITY*

Experiment No.	ATPase units*			Per cent inhibition	
	Control	Digitoxin**	"Peroxide" digitoxin**	Digitoxin	"Peroxide" digitoxin
1.	1.75	1.67	1.53	4	13
2.	1.73	1.43	1.30	17	24
3.	1.53	1.05	0.88	31	42
4.	1.43	1.20	1.10	15	23

*General enzyme reaction conditions, ATPase units, *etc.* are the same as those defined in the footnote for TABLE 2. Original (30 per cent) homogenates in experiments 1 and 2 were centrifuged at 900 × g. Those in experiments 3 and 4 were centrifuged at 580 × g.

**Both digitoxin and "peroxide" digitoxin were added to the original 30 per cent homogenate to effect a concentration of 2.5 μg./ml. (as digitoxin) and incubated for 30 minutes before dilution and immediate addition to the enzyme reaction mixtures. "Peroxide" digitoxin is digitoxin which had been shown to have taken up oxygen by polarographic analysis.

In view of the fact that these experiments had demonstrated that digitoxin uptake of oxygen resulted in the formation of a peroxide product, the production of which was accelerated by the presence of the plasma and cellular constituents of blood in very low concentration, we tested the effect of this "peroxide" glycoside on the sulfhydryl critical enzymes under study. The results of such studies on ATPase and ACHase have been tabulated in TABLES 4 and 5. In each case, the experiments show an increase in the degree of digitoxin inhibition of the enzyme after formation of the "peroxide" digitoxin. It is interesting to note that polarographic estimation of the homogenate sulfhydryl groups using p-chlormercuribenzoate indicated an inverse relationship between available free sulfhydryl and degree of inhibition in the experiments in which such estimation was carried out.

The effect of cysteine on digitoxin inhibition of ACHase and ATPase is presented in the representative experimental results shown in TABLE 6. The findings would seem to support the possibility that an effect on enzyme sulfhydryl is involved in the digitoxin inhibition of the enzymes. The sulfhydryl amino acid effectively reversed the inhibitory action of the digitoxin on the ATPase in the presence of air (experiment A), but a reversal effect of this compound on digitoxin ACHase inhibition could be effected only with nitrogen as the gas phase of the homogenate solution (experiment B). Indeed, from the results obtained in experiments A and C, it is evident that cysteine, in the presence of air, apparently enhances rather than reverses the digitoxin ACHase inhibition. Preliminary polarographic findings utilizing the Bridicka method[30] have indicated that cysteine oxidation to cystine can be effected more readily

TABLE 5

COMPARATIVE EFFECT OF DIGITOXIN AND "PEROXIDE" DIGITOXIN ON RABBIT HEART ACHASE ACTIVITY*

Experiment No.	μM acetylcholine hydrolyzed/30 min.			Per cent inhibition	
	Control	Digitoxin**	"Peroxide" digitoxin**	Digitoxin	"Peroxide" digitoxin
1.	18.4	11.5	9.2	38	50
2.	13.8	9.2	8.2	40	48
3.	18.4	13.8	9.2	25	50
4.	11.5	6.9	4.6	40	60
5.	16.1	9.2	4.6	43	69

*General enzyme reaction conditions are the same as those defined in the footnote for TABLE 1.

**Both digitoxin and "peroxide" digitoxin were added to the homogenate to effect a concentration of 2.5 μg./ml. (as digitoxin) and incubated for 30 minutes before addition to the enzyme reaction mixtures. "Peroxide" digitoxin is digitoxin which had been shown to have taken up oxygen by polarographic analysis.

at pH 7.0 in the presence of dissolved oxygen (air) and digitoxin than with air alone.[28] These observations pose the possibility that heart ACHase may be susceptible to cystine inhibition, and that the cysteine-cystine system, or its analogous derivative systems (*i.e.*, reduced glutathione-oxidized glutathione) may play a role in the glycoside inhibition of ACHase. As no such cysteine enhancement of digitoxin ATPase inhibition was seen in the same homogenates in which it had been demonstrated for digitoxin effect on ACHase (experiment A), it is probably safe to assume that the ATPase is not susceptible to this inhibitory mechanism.

Discussion and Theoretical Considerations

In considering the results obtained from these experiments, it has been recognized from the outset that any interpretation using the findings to correlate with the observed responses of the *in situ* heart to digitoxin is open to the usual limitations that obtain whenever *in vitro* results are correlated with phenomena observed *in vivo*. It would seem that any postulates made would of necessity take the form of a working hypothesis subject to the test of proper analysis of the results from well-designed experiments subsequently performed *in vivo*. With these limitations in mind, such a consideration of the observations has been made.

It is significant that the digitoxin inhibition of the three enzyme systems studied has been observed at concentrations of digitoxin (with reference to homogenate tissue level) that are well below those considered toxic to the *in situ* rabbit heart and that are definitely within the range at

TABLE 6

EFFECT OF CYSTEINE ON DIGITOXIN ENZYME INHIBITION*

Experiment	Condition of 30% Homogenate				Per cent Inhibition	
	Digitox. Conc. μg./ml.	Cysteine Molar Conc.**	Gas Phase	pH	ACHase	ATPase
A.	2.5	0.01	air	7.0	69	0
	2.5	0.10	air	7.0	100	0
	2.5	none	air	7.0	39	10
B.	2.5	0.01	N_2	7.0	16	—
	2.5	none	N_2	7.0	50	—
C.	2.5	0.01	air	7.0	50	—
	2.5	none	air	7.0	33	—

*Enzyme activity determinations carried out according to procedures listed in TABLES 1 and 2, except that incubation of the 30 per cent homogenates with digitoxin was carried out during the last 15 minutes of a total of 30 minutes homogenate incubation period.

**Added to the homogenate 15 minutes before addition of the digitoxin.

which increased heart mechanical efficiency is produced without arrhythmia *in vivo*. The observed inhibitory action of the glycoside on ATPase and ATP deaminase allows for the possibility that it could act to preserve ATP in the ATP deficient myocardium by two different mechanisms of action. In this connection, it should be pointed out that the inhibition of ATPase could be demonstrated consistently only when substrate levels of ATP (2 millimolar final concentration) somewhat lower than those usually used in ATPase reaction mixtures (3 millimolar final concentration) were employed. Not only does this make our findings compatible with the work of others who could find no effect on ATPase (at the higher ATP level), using glycoside concentrations similar to those used by us, but it also presents the theoretical implication that digitoxin effect on ATPase, and, hence, on the preservation of ATP, may be subject to regulation provided by the level of ATP in the myocardium. The experiments carried out to ascertain the nature of the digitoxin ATPase inhibition, which indicated reversible union of ATPase and digitoxin (FIGURE 2) and the competitive nature of the inhibition (FIGURE 3), strongly support this possibility.

The digitoxin inhibition of ACHase has been shown to result from either irreversible or pseudoirreversible union of the digitoxin with the enzyme (FIGURE 4). Under such conditions, the inhibitor virtually "titrates" the enzyme, and the degree of inhibition obtained depends upon the concentration of the enzyme.[29] This finding on the nature of the digitoxin-ACHase dissociation constant is of interest in a consideration of the specificity of digitoxin action. The work of Glick and his co-workers on the distribution of ACHase[31] is pertinent in that they showed that very low levels of ACHase were present in the heart as compared with the high levels found at such sites as the brain, nerve tissue, intestine, *etc.* This distribution of ACHase would imply that a given amount of digitoxin could bring about a pharmacologically significant degree of ACHase inhibition in the heart without exerting sufficient effect on the enzyme at other critical sites (such as the brain and nerve tissue) to manifest observable pharmacological change.

The polarographic studies made on the digitoxin and the "peroxide" digitoxin effects on the sulfhydryl of homogenates from which inhibited ATPase and ACHase were obtained give preliminary indication that the glycoside may be exerting its inhibition by way of an effect on the critical sulfhydryl groups of the enzymes. This is further supported by the reversal of the glycoside effects by cysteine under appropriate conditions. As has been mentioned previously, this reversal is more complicated in the case of ACHase than it is in the case of ATPase. Cysteine "enhancement" of digitoxin ACHase inhibition was observed under

aerobic conditions, while cysteine reversal of the same process was seen under anaerobic conditions. These observations, considered along with the experiments demonstrating digitoxin acceleration of air oxidation of cysteine to cystine, pose the possibility that the glycoside may cause inhibition of a cystine (or cystine derivative) susceptible ACHase either by virtue of its direct action on the enzyme, or by an indirect pathway involving alteration of extra-enzyme cellular sulfhydryl, or both. It is recognized that, due to the nonspecific nature of the enzyme sources (homogenates), none of these experiments conclusively establishes a specific sulfhydryl effect mechanism for the digitoxin inhibitions observed. This would require the use of highly purified enzymes. Nevertheless, the evidence obtained strongly supports such a possibility and certainly presents nothing that is incompatible with it.

In the case of digitoxin inhibition of ATP deaminase, the polarographic demonstration of digitoxin lactone ring complexing of oxygen is considered pertinent. Oxygen has been demonstrated to be a reactant that is critical to the activity of this enzyme. The complexing of oxygen by digitoxin has been shown to require four molecules of the glycoside per molecule of oxygen. Correlation of these findings has led us to postulate that the glycoside ATP deaminase inhibition could be caused by union of the digitoxin with the enzyme followed by digitoxin complexing of oxygen in such a manner as to prevent access of the reactant to its usual critical complexing site on the enzyme.

It is interesting to note that the inhibitions that have been observed *in vitro* can be correlated with the *in vivo* response of the myocardium to digitoxin. Inhibition of ATPase and ATP deaminase spares ATP by minimizing alteration at both ends of the molecule. Such preservation of ATP in the ATP-deficient heart would tend to return the ATP level toward the optimum required for optimal relaxation. By fostering this more optimal relaxation through maintenance of the ATP required for ATP structural contribution to a functional actomyosin,[1,32] the digitoxin could cause increased force of contraction superimposed on optimal diastole in direct obedience to Starling's law. Such increased force of myocardial contraction is a commonly observed response to digitoxin. Digitoxin inhibition of ACHase would spare acetylcholine, which exerts an effect on cardiac impulse conduction exactly analogous to that seen in the presence of the digitalis glycosides. Such sparing of acetylcholine by digitoxin is also compatible with the observations made by others on the changes in heart intracellular electrolyte pattern seen under the influence of acetylcholine[33] on the one hand and in the presence of cardiac glycosides[34] on the other hand. Intracellular loss of potassium and gain in sodium has been shown to be effected by both substances.

It is therefore not too unreasonable to assume that the digitoxin effect on this electrolyte shift might be secondary to its sparing of acetylcholine by inhibition of ACHase.

A postulate involving preservation of ATP by virtue of a cardiac glycoside inhibition of ATPase due to a sulfhydryl mechanism must countenance the existence of the sulfhydryl critical enzymes which are normally involved in ATP synthesis. In the light of present knowledge, it would seem that these enzyme systems, as well as many others involved in the carbohydrate metabolism necessary for synthesis of ATP, are unaffected by the cardiac glycosides.[35-39] It would appear that a selective inhibition by sulfhydryl reaction with specific enzymes (*i.e.*, ACHase and ATPase), to the exclusion of such action on other enzymes, is indeed possible in the case of the cardiac glycosides.

Some reports of research on the cardiac glycosides and some hypothetical considerations could conceivably present barriers to the ultimate validity of the hypothesis that has just been advanced. It is germane to a theoretical consideration of the hypothesis to analyze these potential obstacles. The findings of Wollenberger present such a case. Wollenberger has stated that the failing isolated heart does not have lowered APP levels.[40] He has also reported that ouabain and digoxin cause no change in the APP level of the heart.[39] The opinion that his findings are not necessarily incompatible with the hypothesis advanced by the authors can be reached by critical consideration of his analytical methods. This is especially so in relationship to our own findings on ATP deaminase. All of Wollenberger's determinations of APP were based on seven-minute acid hydrolysis of the nucleotide phosphate. Inosine triphosphate, the deamination product of ATP, cannot be differentiated from ATP under these conditions unless the adenine moiety of the molecule is also determined.[25,41] As Wollenberger made no adenine determinations, the presence of ATP deaminase in heart would leave the accuracy of his findings in considerable doubt.

Another possible impediment to acceptance of the authors' hypothesis is related to the question of whether or not the digitoxin molecule can penetrate the cell membrane in order to gain access to the intracellular enzymes on which its effect is postulated to take place. While we do not believe that this question has been resolved satisfactorily to date, we are also of the opinion that any reasoning which would conclude that digitoxin penetration of the cell is impossible is an extremely dangerous conjecture. At the present time, such a conclusion could be based only upon a rather dubious analogy drawn between the digitoxin molecule and other molecules of similar size that do not penetrate the cell membrane. The most pertinent work on this question that has come to the attention of the authors is the work of Harvey and Pieper.[42] These

investigators showed that when C^{14}-labelled digitoxin was perfused through the isolated guinea pig heart, significant amounts of the radioactivity were found in the mitochondria following ultracentrifugal particulation of the cells. While their experimental method did not distinguish between digitoxin and its possible detoxification products, their results are certainly not inconsistent with the possibility that intracellular distribution of digitoxin does occur.

Summary and Conclusions

1. Inhibition of rabbit heart ACHase and ATPase by digitoxin at homogenate levels of the glycoside that compare favorably to those achieved *in vivo* in cardiac tissue under therapeutic dosage has been demonstrated *in vitro*.

2. The digitoxin complex with ACHase has been shown to be either irreversible or pseudoirreversible. This finding indicates that the degree of digitoxin inhibition of ACHase is regulated by the level of the enzyme. The analogous complex with ATPase has been shown to be reversible. Digitoxin inhibition of ATPase, which has also been shown to be competitive in nature, is subject to regulation by the level of the substrate.

3. Cysteine reversed the digitoxin inhibition of ATPase under aerobic conditions. Cysteine reversed the digitoxin inhibition of ACHase under anaerobic conditions, but seemed to enhance the inhibition under aerobic conditions.

4. Polarographic studies have indicated two effects of digitoxin on dissolved oxygen. The glycoside has been shown to complex molecular oxygen in such a manner as to yield a complex number of four. The digitoxin molecule has also been shown to take up oxygen from solution under certain conditions to form a peroxide product. Formation of this peroxide increases the degree of digitoxin inhibition of ATPase and ACHase.

5. The presence of an ATP deaminase in rabbit heart muscle has been demonstrated. Digitoxin inhibition of this enzyme at homogenate concentrations consistent with therapeutic tissue levels has been observed *in vitro*.

6. Theoretical consideration of these findings has been presented in the form of a hypothesis on the mechanism of digitoxin pharmacological action on the myocardium.

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